

NOVEL MAMMALIAN SECRETED GROUP IIF PHOSPHOLIPASE A₂

RELATED APPLICATION

[0001] This application claims priority of U.S. Provisional Application No. 60/239,491, filed October 11, 2000. This earlier provisional application is hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] This invention relates to DNA and peptide sequences encoding a novel mammalian secreted group IIF phospholipase A₂ and more particularly, a novel human group IIF phospholipase A₂. The invention also relates to the use of this enzyme in methods for screening various chemical compounds.

BACKGROUND

[0003] Phospholipases A₂ (PLA₂, EC 3.1.1.4.) form a superfamily of enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position, producing free fatty acids and lysophospholipids [1-4]. Many intracellular and secreted phospholipases A₂ (sPLA₂s) have been cloned in recent years [2,5], and several of them are involved in a variety of physiological and pathological functions including lipid digestion, cell proliferation, production of lipid mediators of inflammation, antibacterial defense, and cancer [4,6].

[0004] Within the phospholipase A₂ superfamily, sPLA₂s form a relatively homogenous family of enzymes. They are characterized by the presence of several disulfides, an overall conserved three-dimensional structure and a common Ca²⁺-dependent

SUMMARY OF THE INVENTION

[0007] This invention relates to a mammalian secreted group IIF sPLA₂ which is Ca²⁺-dependent, maximally active at pH of about 7-8, and hydrolyzes phosphatidylglycerol versus phosphatidylcholine with about a 15-fold preference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Other advantages and characteristics of the invention will become apparent by reading the following examples concerning the cloning, genomic organization, chromosomal mapping, tissue distribution, and the enzymatic properties of the recombinant hGIIF sPLA₂ and which refer to the attached drawings in which:

[0009] Fig. 1 represents the alignment of the amino acid sequences of human sPLA₂s. Sequences of full-length sPLA₂ proteins are shown. A consensus sequence for the 7 group I/II/V/X human sPLA₂s is presented.

[0010] Fig. 2 represents a schematic diagram of the organization of the human chromosome 1p35 sPLA₂ gene cluster. The total length between *hGIIIE* gene and *hGIIC* pseudogene is about 300 kbp. The PAC clone GenBank n° AL358253 is not yet fully sequenced and the hatched bars indicate the different contigs of this PAC clone. The orientation and exon-intron boundaries of the different sPLA₂ genes are schematically shown. The possible presence of 5' non coding exons in the *hGIIC*, *hGIID*, *hGIIIE* and *hGIIF* genes remain to be determined. The orientation and exact positions of the *hGIIIE* and *hGIIA* genes are unknown. However, based on the sequence of the mouse cosmid KH1 (Genbank n° AC002108) that contains the *mGIIA* gene and a portion of the *mGV* gene, it is likely that the *hGIIA* and *hGV* genes are in a head to tail orientation and that the

hGIIIE gene is localized closer to the telomere.

[0011] Fig. 3 represents the tissue distribution of the human sPLA₂s, as determined by RT-PCR on human adult cDNA panels. The amplified products were analyzed by Southern blot as described in materials and methods. No amplification was observed when cDNA was omitted in the PCR reaction (control lane).

[0012] Figs. 4A-4C represent the enzymatic properties of recombinant hGIIF sPLA₂. Fig. 4(A) shows Ca²⁺-dependence of the hydrolysis of phosphatidylcholine vesicles; Fig. 4(B) shows pH-dependence of the hydrolysis of phosphatidylglycerol vesicles; Fig. 4(C) shows initial velocities for the hydrolysis of the indicated phospholipid vesicles. Full experimental details are provided in materials and methods.

DETAILED DESCRIPTION

[0013] This invention concerns the cloning, tissue distribution and recombinant expression in *E. coli* of a novel mammalian group IIF sPLA₂, more particularly, a novel human group IIF (hGIIF) sPLA₂. This group II sPLA₂ has unique structural features including a long, proline-rich C-terminal extension with an odd cysteine, and a very low pI value. It also has a specific tissue distribution and a fairly high propensity to hydrolyze POPC versus POPG as compared to the other sPLA₂s. Furthermore, using sequences generated by the Human Genome Project, the gene for hGIIF sPLA₂ maps to chromosome 1 together with 5 other sPLA₂ genes to form a sPLA₂ gene cluster that spans about 300 kilobase pair (kbp). Interestingly, 5 of these 6 genes code for group II enzymes and share relatively high level of identity. The last gene coding for group V sPLA₂ is, in fact, also related to group II sPLA₂ genes, as group V sPLA₂ does not contain a propeptide sequence

and displays higher levels of identity to group II sPLA₂s than to groups IB and X sPLA₂s. It is thus likely that these 6 different genes have arisen from recent and successive gene duplication events. It should be also noted that group IIA, IIC, IID, IIE and V sPLA₂s are all basic enzymes while group IIF is very acidic. On the other hand, group IB and X sPLA₂s appear more divergent in sequence and are located on different chromosomes [13]. Both contain a propeptide sequence and the group I specific disulfide bond between cysteines 11 and 77. Whether group IB, X or one of the group II-like sPLA₂s is more related to the sPLA₂ ancestor gene of the group I/II/V/X sPLA₂ collection [5] remains to be determined.

[0014] Thus, the invention concerns a novel mammalian secreted group IIF sPLA₂ wherein the enzyme is Ca²⁺-dependent, maximally active at pH of about 7-8, and hydrolyzes phosphatidylglycerol versus phosphatidylcholine with a about 15-fold preference.

[0015] The invention concerns more particularly a mammalian secreted group IIF sPLA₂ constituted by or comprising the sequence of amino acids in the list of sequences under SEQ ID N°2. More particularly, the mammalian secreted group IIF sPLA₂ is a human secreted group IIF sPLA₂.

[0016] The invention concerns a nucleic acid molecule comprising or constituted of an encoding nucleic sequence for a mammalian secreted group IIF sPLA₂ or for a fragment of a mammalian secreted group IIF sPLA₂ whose amino acid sequence is represented under SEQ ID N°2. The invention relates more particularly to a nucleic acid molecule constituted by or comprising the sequence under SEQ ID N°1. The invention also concerns nucleotide sequences derived from the above sequence, for example, from the degeneracy

of the genetic code or by the suppression or insertion of nucleotides (such as introns), and which encode for proteins presenting characteristics and properties of group IIF sPLA₂.

[0017] The invention also relates to polyclonal or monoclonal antibodies directed against one secreted group IIF sPLA₂ of the invention, a derivative or a fragment of these. These antibodies can be prepared by the methods described in the literature. According to prior art techniques, polyclonal antibodies are formed by the injection of proteins, extracted from animal tissues or produced by genetic transformation of a host, into animals, and then recuperation of antiserums and antibodies from the antiserums for example by affinity chromatography. The monoclonal antibodies can be produced by fusing myeloma cells with spleen cells from animals previously immunized using the proteins of the invention. These antibodies are useful in the search for new secreted mammalian group IIF sPLA₂ or the homologues of this enzyme in other mammals or again for studying the relationship between the secreted group IIF sPLA₂ of different individuals or species.

[0018] The invention also concerns a vector comprising at least one molecule of nucleic acid above, advantageously associated with adapted control sequences, together with a production or expression process in a cellular host of a mammalian group IIF sPLA₂ of the invention or a fragment thereof. The preparation of these vectors as well as the production or expression in a protein host of the invention can be carried out by molecular biology and genetic engineering techniques well known in the art.

[0019] An encoding nucleic acid molecule for a mammalian secreted group IIF sPLA₂ or a vector according to the invention can also be used to transform animals and establish a line of transgenic animals. The vector used is chosen in function of the host

into which it is to be transferred; it can be any vector such as a plasmid. Thus, the invention also relates to cellular hosts expressing mammalian secreted group IIF sPLA₂ obtained in conformity with the preceding processes.

[0020] The invention also relates to nucleic and oligonucleotide probes prepared from the molecules of nucleic acid according to the invention. These probes, marked advantageously, are useful for hybridization detection of similar group IIF sPLA₂ in other individuals or species. According to prior art techniques, these probes are put into contact with a biological sample. Different hybridization techniques can be used, such as Dot-blot hybridization or replica hybridization (such as the Southern technique) or other techniques (such as DNA chips). Such probes constitute the tools making it possible to detect similar sequences quickly in the encoding genes for group IIF sPLA₂ which allow study of the presence, origin and preservation of these proteins. The oligonucleotide probes are useful for PCR experiments, for example, to search for genes in other species or with a diagnostic aim.

[0021] The secreted phospholipases A₂ (sPLA₂) are Ca²⁺-dependent, disulfide-rich, 14-18 kDa enzymes that catalyze the hydrolysis of phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids. sPLA₂s are also ligands that bind to a collection of soluble and membrane bound proteins which are likely to play a role in the biological functions of these enzymes. In the last few years, a number of structurally distinct mammalian sPLA₂s have been identified, and it has become clear that these sPLA₂s are expressed in a variety of tissues under both normal and pathological conditions (including inflammatory diseases, cancers, cardiac and brain ischemia, etc...), and are involved in a myriad of physiological and pathological roles. In mammalian cells stimulated with

proinflammatory agonists, a subset of sPLA₂s play a role in the release of arachidonic acid for eicosanoid production. sPLA₂s are also involved in cell proliferation, cell migration, angiogenesis, cell contraction, apoptosis, neurosecretion, blood coagulation, adipogenesis, lipid metabolism (digestion, skin lipid barrier and lung surfactant formation, lipoprotein metabolism, etc...), spermatogenesis, fecondation, and embryogenesis. They also play a role in host defense and have antiviral and antibacterial properties against viruses like HIV-1 and various Gram-positive and Gram-negative bacterial strains. They also have antitumoral properties. They are also involved in various pathological conditions such as acute lung injury, acute respiratory distress syndrome, Crohn's disease, and various types of cancers where sPLA₂s can act as gene suppressors.

[0022] The invention concerns pharmaceutical compositions comprising as an active agent at least an encoding nucleic acid molecule for a mammalian secreted group IIF sPLA₂, or one molecule for a mammalian secreted group IIF sPLA₂ or a derivative of this protein. These pharmaceutical compositions can be used to treat or prevent viral and bacterial infections. They also can be used to treat or prevent cancers.

[0023] The invention is also useful in methods for identifying biologically active compounds with anti-inflammatory properties or more generally for identifying compounds that modulate sPLA₂ biological activities as listed above.

[0024] Such biologically active compounds can be identified by determining if a selected compound is capable of inhibiting the catalytic activity of sPLA₂ in cleaving a phospholipid to release fatty acids and lysophospholipids in a mixed micelle assay, a liposome assay, a system utilizing natural membranes, or in whole cells overexpressing this enzyme. A compound capable of inhibiting sPLA₂ catalytic activity may have anti-

inflammatory or may behave as an antagonist of sPLA₂ in the sPLA₂ biological activities listed above.

[0025] For example, screening of compounds for potential anti-inflammatory activity can be performed with the novel sPLA₂ enzymes of this invention, purified to homogeneity from cell sources or produced recombinantly or synthetically. A selected compound may be added to a sPLA₂ enzyme of this invention in a mixed micelle assay, a liposome assay, or an assay system utilizing natural membranes and analyzed for inhibition of sPLA₂ activity. Alternatively, a selected compound may be added to whole cells which overexpress the sPLA₂ and the cells examined for inhibition of release of fatty acids or lysophospholipids. In this case, normal cells and cells overexpressing sPLA₂ can be cultured in labeled arachidonic acid. Signal is measured between the secreted products of both the normal and overexpressing cells to provide a baseline of sPLA₂ expression. A selected compound is then added to cultures and the cultures are grown in labeled arachidonic acid. If there is a difference in the signal (e.g., the amount of arachidonic acid produced) in the cells in the presence of the compound, this compound inhibits sPLA₂ activity and may be a potential anti-inflammatory compound.

[0026] Biologically active compounds can also be identified by screening the selected compounds for their binding properties to sPLA₂ receptors that bind group IIF sPLA₂s of this invention. These receptors include the family of N-type and M-type receptors which are likely to be involved in several biological activities of sPLA₂s including HIV-1 antiviral properties. For example, radioactively or fluorescently labeled sPLA₂s can be used in competition binding assays and selected compounds can be screened for inhibition of sPLA₂ binding.

[0027] Biologically active compounds can also be identified by screening the selected compounds for modulation of a sPLA₂ biological effect such as those listed above. For example, sPLA₂s of this invention may be added to cells in the presence or absence of a selected compound and cells may be assayed for cell proliferation, cell migration, cell contraction or apoptosis.

[0028] In general, another aspect of this invention is thus related to the use of a compound first identified by the methods described above. Novel pharmaceutical compositions may contain a therapeutically effective amount of a compound identified by an above method of this invention. These pharmaceutical compositions may be employed in methods for treating disease states or disorders involving group IIF sPLA₂s of this invention.

I. Materials and methods.

I.1 Molecular cloning of hGIIF sPLA₂.

[0029] Searching for sPLA₂ homologs in gene databases stored at the National Center for Biotechnology using the tBLASTn sequence alignment program [20] resulted in the identification of a human genomic sequence (PAC clone dJ169023, GenBank accession number AL158172) of 142849 nucleotides containing several regions of homology with mouse group IIF sPLA₂. A set of oligonucleotides was designed from this genomic sequence (sense primer 5'-ATGAAGAAGTTCTTCACCGTGGCCA-3' (SEQ ID N°3 in the list of sequences in the appendix) and reverse primer 5'-ACCCTCCTCCC-GCTCTCTCTCTCAAA-3'(SEQ ID N°4 in the list of sequences in the appendix)) and used in RT-PCR experiments on different human cDNAs. A DNA product of the expected size was amplified from human cDNAs from spleen, heart, and fetal lung. Sequencing of

the DNA fragments revealed complete identity with the genomic sequence after its appropriate splicing according to consensus exon-intron boundaries [21].

I.2 Tissue distribution of human sPLA₂s.

[0030] Multiple Tissue cDNA Panels (Clontech, catalog n° K1420-1 and K1421-1) were used as templates in RT-PCR experiments using primers specific for the human sPLA₂s cloned so far. PCR reactions were analyzed by agarose gel electrophoresis, transferred to positively charged nylon membranes, and hybridized with specific ³²P-labeled internal oligonucleotide probes.

I.3 Recombinant expression of hGIIF sPLA₂.

[0031] The preparation of a truncated GST hGIIF sPLA₂ construct, bacterial induction and preparation of sulfonated protein from inclusion bodies were carried out as previously described for mouse group IID sPLA₂ [7]. The hGIIF fusion protein was refolded by a rapid dilution method as follows. Sulfonated protein was dissolved to 10 mg/ml in 4 ml of 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, and added dropwise (~ 1 drop per second) to 2 liters of refolding buffer (50 mM Tris-HCl, pH 8.0, 0.9 M guanidine-HCl, 10 mM CaCl₂, 5 mM freshly added cysteine, 30% acetonitrile) with constant stirring at room temperature. Stirring was continued for a few minutes, and then the solution was allowed to sit without stirring at room temperature for ~2-3 days. The sPLA₂ enzymatic activity was monitored with the fluorimetric assay [16] until the activity increase starts to level off. After addition of 5 mM lauryl sulfobetaine (dodecyldimethyl-3-sulfopropylammonium, inner salt) and 1 mM methionine, the protein solution was concentrated by ultrafiltration to 50 ml with a YM-10 membrane (Amicon) and dialyzed 3 times against cleavage buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM CaCl₂).

Freshly made TPCK-treated trypsin (Sigma) was then added to a final concentration of 0.15 $\mu\text{g/ml}$, and the mixture was incubated overnight at room temperature, leading to a ~ 200 -fold increase in sPLA₂ activity. The reaction mixture was directly loaded at 3 ml/min on a Vydac 218 TP1010 C18 reverse phase column equilibrated with solvent A (20% acetonitrile, 0.1% trifluoroacetic acid, 1 mM methionine). Elution was performed at 3 ml/min using a linear gradient (0-6.3% B over 2 min, followed by 6.3-27.5% B over 42 min) of solvent B (100% acetonitrile, 0.1% trifluoroacetic acid, 1 mM methionine). HPLC purified hGIIF sPLA₂ was neutralized with 2 M Tris base, 5 mM lauryl sulfobetaine was added and the sample was concentrated in a Centriprep-10 (Amicon). The protein was then dialyzed against 10 mM Tris pH 8.0, 0.1 mM DTT, at 4 °C for 1 cycle to cleave the disulfide between the cysteine in the C-terminal extension of the hGIIF sPLA₂ and free cysteine from the refolding buffer, and then against 10 mM Tris-HCl, pH 8.0 for two cycles. The approximate yield of final product per liter of *E. coli* culture is 3.7 mg. Concentrations of recombinant hGIIF sPLA₂ were determined by OD at 280 nm using an extinction coefficient of 10.37 calculated from the amino acid sequence.

I.4 sPLA₂ catalytic activity studies.

[0032] The Ca²⁺ and pH dependencies of hGIIF sPLA₂ were measured with POPC vesicles containing 1-palmitoyl-2-[1-³H]palmitoyl-*sn*-glycero-3-phosphocholine vesicles and POPG vesicles containing 1-palmitoyl-2-[1-³H]palmitoyl-*sn*-glycero-3-phosphoglycerol, respectively [7]. Substrate specificity studies were carried out using a slightly modified assay with the fatty acid binding protein [7]. Reaction mixtures contained 30 μM POPC, POPG, or POPS large unilamellar vesicles (0.1 μm , prepared by extrusion as described [22]) in Hanks' balanced salt solution with 1 mM Ca²⁺, 1 mM Mg²⁺, 9.7 μg fatty acid

binding protein, and 1 μ M 11-dansyl-undecanoic acid at 37 °C. Assays were calibrated by adding a known amount of oleic acid to the complete assay in the absence of enzyme.

II. Results and discussion.

II.1 Cloning of human group IIF sPLA₂.

[0033] Screening of nucleic sequence databases with various mouse sPLA₂s led to the identification of a large human genomic fragment of 142849 nucleotides with several regions of homology to mouse group IIF (mGIIF) sPLA₂ [8]. It was thus likely that this genomic clone contains a complete gene with several exons and introns coding for human group IIF sPLA₂. Based on homology with mGIIF, a set of sense and antisense primers was designed from the genomic sequence to amplify the full-length hGIIF sPLA₂ cDNA by RT-PCR. Human cDNAs from spleen, heart and fetal lung were used, and a strong amplification was obtained with spleen cDNA. The sequences of the amplified DNA fragments were found to contain an open reading frame of 168 amino acids comprising a signal peptide of 20 residues followed by a mature protein sequence of 148 residues (Fig. 1). This sequence is 74% identical to mGIIF sPLA₂ and contains all of the structural features of mGIIF, including the very long C-terminal extension of 23 amino acids [8]. Together, these results indicate that the 168 amino acid sequence corresponds to hGIIF sPLA₂ (Fig. 1).

[0034] The hGIIF mature protein sequence (calculated molecular mass 15,598 Da) is the most acidic sPLA₂ identified so far in mammals, with a calculated pI of 4.51. The 23 amino acid C-terminal extension of hGIIF also appears relatively acidic, as it contains 3 glutamic acid residues and no basic residues. Furthermore, one third (8 out 23) of the residues of this C-terminal extension are proline residues. Interestingly, these specific

features appear to be conserved among species, as the mouse group IIF C-terminal sequence is also acidic and proline-rich. The odd cysteine residue found in the mGIIF sPLA₂ C-terminal extension is also conserved in the hGIIF sPLA₂ sequence. The possible involvement of these amino acids in the putative homo- or heterodimerization of group IIF sPLA₂s remains to be determined. Four potential N-glycosylation sites were found in the mature sequence of hGIIF sPLA₂ at positions 79, 89, 110 and 134 (Fig. 1) and only three of them (positions 79, 89 and 134) are conserved in the mGIIF sequence [8].

[0035] An alignment of the amino acid sequences of the 7 human catalytically-active group I/II/V/X sPLA₂ collection is presented in Fig. 1, and their respective levels of identity is shown in Table I. hGIIF sPLA₂ contains the different residues which are conserved in all catalytically active sPLA₂s and is particularly well-conserved with other human sPLA₂s in the Ca²⁺ loop and the active site domains. hGIIF sPLA₂ however shows low levels of identity with other human sPLA₂s, and the most closely related sPLA₂ is hGIID with only 41 % identity (Table I), indicating that hGIIF sPLA₂ is not an isoform of the previously cloned human sPLA₂s. It should be noted that the highest level of identity between any two sPLA₂s is observed between GIIA and GIIE (55 % of identity in human species (Table I) and 51 % in mouse species [8].

Table I: Level of amino acid sequence identity (%) between the different human sPLA₂s

sPLA ₂	hGIIA	GIID	GIIE	hGIIF	hGV	hGX
hGIB	35	36	35	27	30	29
hGIIA		50	55	33	44	35
hGIID			39	41	42	39
hGIIE				35	41	38
hGIIF					33	29
hGV						37

II.2 hGIIF sPLA₂ gene maps to chromosome 1 and belongs to a sPLA₂ gene cluster.

[0036] We have previously reported that the six genes for mGIIA, mGIIC, mGIID, mGIIE, mGIIF and mGV sPLA₂s are located in the distal part of mouse chromosome 4 and most likely form a sPLA₂ gene cluster [8]. Furthermore, the genes for hGIIA, hGIIC and hGV sPLA₂s have also been proposed to form a gene cluster positioned between the genetic markers AFM217zc3 and AFM290vb9 [14]. Here, we have taken advantage of the human genome sequencing project to show that the 6 corresponding human sPLA₂ genes are in fact located very close to each other within a DNA fragment of about 300 kbp. The organization of the sPLA₂ gene cluster is presented in Fig. 2.

[0037] The human PAC clone dJ169023 (GenBank n° AL158172) of 141,865 bp that contains the *hGIIF* gene was generated by the sequencing program of human chromosome 1, assigning the *hGIIF* gene to this chromosome. In addition to the *hGIIF* gene, this PAC clone contains also the full-length genes for *hGV*, *hGIID*, as well as the *hGIIC* pseudogene. The *hGIIA* and *hGIIE* genes were found to be localized on the overlapping PAC clone dJ169023 (GenBank n° AL358253) in the telomeric direction (Fig.

2). At present, the available sequence of this PAC clone is composed of 49 unordered contigs of different lengths. Based on alignments of these different contigs with the sequence of the PAC clone AL158172, the overlapping sequence between AL158172 and AL358253 is estimated to be about 28 kbp. The relative orientation of *hGIIA* and *hGIIE* with the other sPLA₂ genes and the exact distances between *hGIIA* and *hGIIE* genes, and *hGIIA* and *hGV* genes are unknown. However, based on the full-length sequence of the mouse cosmid clone of 41,125 bp (GenBank AC002108) that contains the *mGIIA* gene and a portion of the *mGV* gene [23], it is likely that the *hGIIA* and *hGV* genes are organized in a head to tail orientation and that the *hGIIE* gene is localized in the telomeric direction, as presented in Fig. 2.

II.3 Analysis of the tissue distribution of hGIIF sPLA₂.

[0038] The tissue distribution of hGIIF and other human sPLA₂s was analysed by RT-PCR experiments using commercial human cDNA panels. As shown in Fig. 3, hGIIF sPLA₂ is expressed at high levels in placenta, testis, thymus, and at lower levels in heart, kidney, liver and prostate. Very low signals were observed in skeletal muscle, pancreas, small intestine and spleen. In the mouse species, mGIIF transcripts were detected mostly in testis but also in several other tissues [8]. In the future, it will be interesting to analyse the expression of hGIIF sPLA₂ in embryos, since high levels of mGIIF transcripts were observed at different stages of embryonic development [8]. The different patterns of expression of human sPLA₂s presented in Fig. 3 and previously found for hGIIE sPLA₂ [11] clearly indicate that all human sPLA₂s including hGIIF most probably have non redundant functions. Overall, the tissue distributions of the human sPLA₂s resemble those previously found by northern-blot analysis [12,13,15]. Finally, it is interesting to note that

all human sPLA₂s are expressed in the pancreas and that placenta, lung, colon and small intestine are also rich sources of sPLA₂.

II.4 Recombinant expression of hGIIF sPLA₂.

[0039] In order to study the interfacial kinetic properties of hGIIF sPLA₂, we produced this enzyme as a recombinant fusion protein in *E. coli*. Inclusion bodies containing hGIIF fusion protein were solubilized and reduced, and free cysteines were sulfonated. Rapid dilution of the sulfonated protein into a buffer containing 30% acetonitrile, to minimize protein aggregation, produced refolded fusion protein which displayed maximal activity after 2-3 days. The fusion protein contains a factor Xa recognition site adjacent to the N-terminal residue of mature hGIIF which could be efficiently cleaved by using Factor Xa and trypsin. Cleaved hGIIF sPLA₂ was purified to homogeneity by chromatography on a C18 reverse phase column, and the overall yield of purified hGIIF sPLA₂ is 3.7 mg per liter of *E. coli* culture (data not shown).

[0040] The interfacial enzymatic properties of hGIIF sPLA₂ are summarized in Fig. 4. The hydrolysis of phospholipid vesicles by hGIIF sPLA₂ is strictly Ca²⁺ dependent, as expected for a typical sPLA₂. Using PC vesicles as substrate, the enzyme displayed a hyperbolic dependence on the concentration of Ca²⁺ (Fig. 4A), and an apparent K_{Ca}²⁺ of 140 ± 40 μM was calculated. Fig. 4B shows that the rate of hydrolysis of phosphatidylglycerol vesicles by hGIIF sPLA₂ increases with pH in the range 5-7, as expected from the deprotonation of the active site histidine residue, and then decreases slightly at pH above 7. The relative rates for the hydrolysis of POPG, POPS, and POPC vesicles by hGIIF sPLA₂ are compared in Fig. 4C. As for all mammalian sPLA₂s examined so far [7,8], the enzymatic activity of hGIIF sPLA₂ is highest with anionic

POPG vesicles, which probably reflects the relatively high affinity of all sPLA₂s for POPG vesicles. Although hGIIF hydrolyzes POPC at only ~6% of the rate of POPG, this enzyme is much more active on POPC vesicles than hGIIA sPLA₂, which displays a greater than 10⁵-fold preference for POPG versus POPC vesicles [8]. In this context, hGIIF appears more similar to hGV and hGX sPLA₂s, which are 3- and 10-fold more active on POPG versus POPC vesicles, respectively [16]. Whether exogenous hGIIF sPLA₂, like hGX sPLA₂, is able to efficiently release arachidonic acid from adherent cells will be interesting to analyse [16].

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